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Protein disulphide isomerase as a target for nanoparticle-mediated sensitisation of cancer cells to radiation

L E Taggart¹, S J McMahon^{1,3}, K T Butterworth¹, F J Currell²,
G Schettino^{1,4} and K M Prise¹

¹ Centre for Cancer Research and Cell Biology, Queen's University Belfast, BT9 7AE, UK

² School of Mathematics and Physics, Queen's University Belfast, BT7 1NN, UK

³ Department of Radiation Oncology, Massachusetts General Hospital, 30 Fruit St, Boston, MA 02114, USA

⁴ National Physical Laboratory, Teddington, TW11 0LW, UK

E-mail: ltaggart04@qub.ac.uk

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Abstract

Radiation resistance and toxicity in normal tissues are limiting factors in the efficacy of radiotherapy. Gold nanoparticles (GNPs) have been shown to be effective at enhancing radiation-induced cell death, and were initially proposed to physically enhance the radiation dose deposited. However, biological responses of GNP radiosensitization based on physical assumptions alone are not predictive of radiosensitisation and therefore there is a fundamental research need to determine biological mechanisms of response to GNPs alone and in combination with ionising radiation. This study aimed to identify novel mechanisms of cancer cell radiosensitisation through the use of GNPs, focusing on their ability to induce cellular oxidative stress and disrupt mitochondrial function. Using N-acetyl-cysteine, we found mitochondrial oxidation to be a key event prior to radiation for the radiosensitisation of cancer cells and suggests the overall cellular effects of GNP radiosensitisation are a result of their interaction with protein disulphide isomerase (PDI). This investigation identifies PDI and mitochondrial oxidation as novel targets for radiosensitisation.

Online supplementary data available from stacks.iop.org/NANO/27/215101/mmedia

Keywords: nanoparticle, bioactivity, thiol, oxidation

(Some figures may appear in colour only in the online journal)

Introduction

Radiotherapy aims to maximise the differential between radiation dose deposited in the tumour whilst minimising the dose to the surrounding normal tissue. Although clinical delivery methods such as IMRT and hadron therapy have significantly improved conformity to the tumour target, the

potential for radiation sensitisers to increase the therapeutic index of radiotherapy remains to be developed.

One of the main challenges in radiotherapy and tumour imaging is the lack of contrast between the radiation absorption properties of normal tissue and cancerous tissue making normal tissue tolerance the limiting dose factor in the delivery of radiotherapy [1]. Creating a contrast between these two tissues would reduce the size of the delivered dose outside the target, sparing normal tissue toxicity, whilst achieving the effects of a higher dose in the cancerous tissue. High Z (atomic number) materials such as gadolinium, iodine and gold to name a few, have the potential to physically



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enhance the local dose deposited by the radiation beam as a result of favourable mass attenuation coefficients [2]. An enhanced contrast between two materials resulting in additional energy deposition was first demonstrated by Spiers in 1949 when he investigated why tolerances of soft tissue to radiation was dependent on its vicinity to bone [3].

Considering atomic number, gold is an obvious candidate as a contrast agent and radiation modifier. In the form of nanoparticles, gold has a range of potential properties for use in cancer therapy is significantly enhanced with the ability to specifically target GNPs to tumour cells while delivering anti-cancer drugs. High *Z* number materials are characterised by the large numbers of electrons in with high photoelectric cross-sections, resulting in high mass attenuation coefficients. As a result, high *Z* materials have the potential to physically enhance the local dose deposited by the radiation beam. High *Z* materials such as iodine have been used as imaging contrast agents for decades, although early studies showed *in vitro* that these agents are capable of causing additional cell damage following irradiation [4, 5]. Recent *in vivo* studies have observed evidence of increased damage when these agents are present during imaging [6–8]. Although safer contrast agents would be beneficial, in cancer treatment it is anticipated that the conventionally undesirable effects of these contrast agents can be taken advantage of to improve tumour cell killing.

Gold nanoparticles (GNPs) and nanotechnology are not new concepts but their applications in biology and particularly medicine offer innovative options for future applications. In particular, their potential for use in cancer therapy offers a multi-use treatment which could enhance tumour imaging and cancer cell targeting as well as cancer drug delivery. GNPs have been shown to target tumour cells passively as well as having the potential to be modified with targeting molecules such as antibodies to actively target tumour cells [9]. In passive targeting, the GNPs take advantage of the enhanced permeability and retention effect which allows them to migrate through the leaky vasculature cultivated by tumour cells and thereby preferentially accumulate at tumour sites [10]. Alternatively GNPs can be conjugated with a targeting molecule, typically an antibody, which can detect a tumour specific molecule which is overexpressed on the tumour cell such as EGFR, and ideally this targeted molecule should be vital for cellular function [10, 11].

We have previously calculated that a 1% weight for weight concentration of gold would double the absorbed dose at kilovoltage energies [1]. Calculations have predicted dose enhancement factors (DEFs) of between 1.2 and 5 when using GNPs in combination with radiation depending on the quantity of gold present and the energy of the beam used [12, 13]. These studies suggest that dose enhancement is most achievable in the kilovoltage energy range due to the strong contrast between soft tissue and GNPs in this energy region [13, 14]. By contrast, their similar absorption at megavoltage energies leads to limited contrast in higher energy exposures. Most clinical radiotherapy equipment operates outside the predicted optimal range, using megavoltage energies with filters to reduce the low energy (keV) component.

Despite these predictions radiosensitization has been shown in cells exposed to GNPs and irradiated with megavoltage energies, and at concentrations significantly below the 1% mass suggested by theoretical predictions [15, 16]. While radiosensitization observed at these higher energies can be partly explained by considering the shower spectrum produced in the sample and the interaction of the daughter particles with the nanoparticles [17], there remains the possibility of additional, perhaps biological, processes in the radiosensitising effect of GNPs. This was highlighted in a recent review by Butterworth *et al* who concluded that the physical dose deposition model did not accurately reflect or explain the observed experimental outcomes of GNPs with radiation in cellular systems [2]. Numerous potential mechanisms were identified, including increased cellular oxidative stress, impacts on cell cycling or DNA repair, and issues around nanoparticle toxicity.

A large number of studies have sought to determine mechanisms of cellular responses to GNPs whilst comparatively few have investigated biological sensitisation effects of GNPs in combination with irradiation. Several previous reports from our group have focussed on the commercially available 1.9 nm AurovistTM GNP (Nanoprobes Inc.) [15, 18–20]. This study aimed to further these previously published studies in order to determine the biological effects of these nanoparticles and elucidate the mechanism of GNP radiosensitization. We have already shown radiosensitisation, an oxidative stress response as well as DNA damage in cells after treatment with these GNPs. This paper furthers this previous research by linking the observed effects and identifying a novel biological mechanism which is a significant driver for the observed radiosensitisation of cancer cells by these widely studied GNPs.

Materials and methods

Cell culture

All cell lines were obtained from Cancer Research UK. The human breast cancer cell line, MDA-MB-231 was maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. The human prostate cell line, DU-145 was maintained in RPMI-1640 medium with 10% foetal bovine serum and 1% penicillin/streptomycin. The human glioma cell line, T98G was maintained in Eagle's minimum essential media supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. All cells were grown at 37 °C in a humidified atmosphere of 95% air/5% CO₂. N-acetyl-Cysteine (catalogue no. 7250, Sigma-Aldrich, St Louis, MO) was dissolved in H₂O and neutralised. Cells were treated at a concentration of 5 mM for 2 h prior to or after GNP incubation. Bacitracin (catalogue no. B0125, Sigma-Aldrich) was dissolved in H₂O and cells were treated at 3 mM for 2 h (prior to incubation with GNPs. AurovistTM 1.9 nm GNPs were purchased from Nanoprobes Inc. (NY) and resuspended in H₂O. Cells were

treated at a concentration of $500 \mu\text{g ml}^{-1}$ for 24 h unless otherwise indicated.

Detection of reactive oxygen species (ROS)

Cells were seeded into 12 well plates at a density of 1×10^5 cells per well and left to attach for 4–6 h before being treated accordingly. ROS were measured using Carboxy-H2DCFDA (Invitrogen Life Technologies, cat no. C13293) at a concentration of $5 \mu\text{M}$. Cells were incubated with the dye in serum free OptiMem Media (Invitrogen Life Technologies) for 45 min, then cells were allowed to recover for 30 min in normal serum media, 2.5 mM hydrogen peroxide was added to positive control cells at this stage. Cell media was collected and placed in 15 ml centrifuge tubes while cells were detached using 0.1% EDTA in PBS and added to the collected media. Cells were then pelleted by centrifugation at 2000 rpm at 4°C for 5 min. Media was removed and cell pellets were suspended in PBS and analysed immediately using a FACSCalibur flow cytometer and CELL-Quest software (Becton-Dickson). 1×10^4 cells were analysed per sample.

Mitochondrial oxidation detection

Mitochondrial oxidation was measured using Nonyl-Acridine Orange (NAO) (cat no A-1372, Molecular Probes, Invitrogen, NY). 1×10^5 cells were seeded into 12 well plates and left to attach for 4–6 h before being treated accordingly. At the end of treatment, media was removed from cells and transferred to 15 ml centrifuge tubes on ice. Cells were detached using 0.25% Trypsin/ 1 mM EDTA solution and added to corresponding tubes containing media. Cells were then pelleted by centrifugation at 2000 rpm at 4°C for 5 min. Media was removed and cell pellets were resuspended in $300 \mu\text{l}$ of 0.1% BSA-PBS solution containing 25 ng ml^{-1} NAO and left to incubate at 37° for 10 min. Cells were placed on ice post-incubation and analysed immediately using FACSCalibur flow cytometer and CELL-Quest software (Becton-Dickson). 1×10^4 cells were analysed per sample.

Mitochondrial membrane polarisation measurement

Cells were seeded into 12 well plates at a density of 1×10^5 cells per well and left to attach for 4–6 h before being treated accordingly. 25 nM Tetramethylrhodamine ethyl ester perchlorate (TMRE) (Sigma-Aldrich) was added to each well and incubated for 15 min at 37°C . Media was then transferred to 15 ml centrifuge tubes and placed on ice. Cells were detached using 0.25% trypsin and 1 mM EDTA and the cell solution was then transferred to the corresponding 15 ml tube left on ice. Cells were then pelleted by centrifugation at 2000 rpm at 4°C for 5 min. Media was removed and cell pellets were resuspended in $300 \mu\text{l}$ of PBS and TMRE fluorescence was analysed immediately using a FACSCalibur flow cytometer and CELL-Quest software (Becton-Dickson). 1×10^4 cells were analysed per sample.

Clonogenic cell survival assay

Sub-confluent cells were removed from flasks using a solution of 0.25% Trypsin and 1 mM EDTA, they were counted using a coulter counted and re-seeded into six well plates at a density of 1.5×10^5 cells per well. Cells were left to attach for 4–6 h and were then treated with GNPs for 24 h. Cells were then irradiated, trypsinised and counted, then seeded into T25 flasks using appropriate cell numbers and left to proliferate for 7–9 d.

Statistical analysis

Cell survival data was fitted with a linear quadratic trend, $S = e^{-\alpha D - \beta D^2}$, where α and β are radiosensitivity parameters and D is the delivered dose and fitted using the nonlinear regression function in Prism version 4.01 (Graphpad Software Inc.). DEF is defined here as the ratio of doses which lead to equal levels of cell survival with and without GNPs. DEFs can vary with delivered dose, and are quoted with reference to the dose delivered to cells in the absence of GNPs. Statistically significant differences were calculated using the two tailed paired t-test function in Prism version 4.01 with a p value of ≤ 0.05 considered as significant.

Results

Cellular effects of GNP treatment

MDA-MB-231, DU145 and T98G cells were treated with 1.9 nm GNPs at a concentration of $500 \mu\text{g ml}^{-1}$ for time intervals ranging from 1 to 24 h. These nanoparticles, the concentration, these time-points and the cell lines were chosen as a result of previous work within the group showing that $500 \mu\text{g ml}^{-1}$ for 24 h allows for maximal cell uptake [15]. Preliminary data were also obtained with a 15 nm GNP from Nanoprobes, however no significant sensitisation was observed in these particles (supplementary figure 1). This absence of an effect is believed to be the result of their different surface functionalization to the 1.9 nm GNPs, and so these particles were not taken forward to subsequent experiments. The cell lines used represent tumours from different sites of the body with varying degrees of radiosensitivity. 1.9 nm GNP radiosensitisation was also tested in normal human fibroblast cells, AGO-1522b, but no radiosensitisation was observed (supplementary figure 2). At each time-point, levels of ROS, mitochondrial polarisation and mitochondrial oxidation were measured post incubation (figure 1). All cell lines responded with a 2 to 3 fold increase in ROS levels from the 1 h time-point which were maintained until the 24 h time-point in both MDA-MB-231 and DU145 cell lines, however only the 1 and 24 h increases in ROS in the MDA-MB-231 cells were significant (figure 1(A)). A trend for increased ROS of up to 5 fold was observed in T98G cells at the 1, 3 and 24 h time-points with the 6 h time-point showing ROS levels closer to those seen in the control cells.

Mitochondrial membrane polarisation was assessed by measuring the retention of tetramethylrhodamine ethyl ester

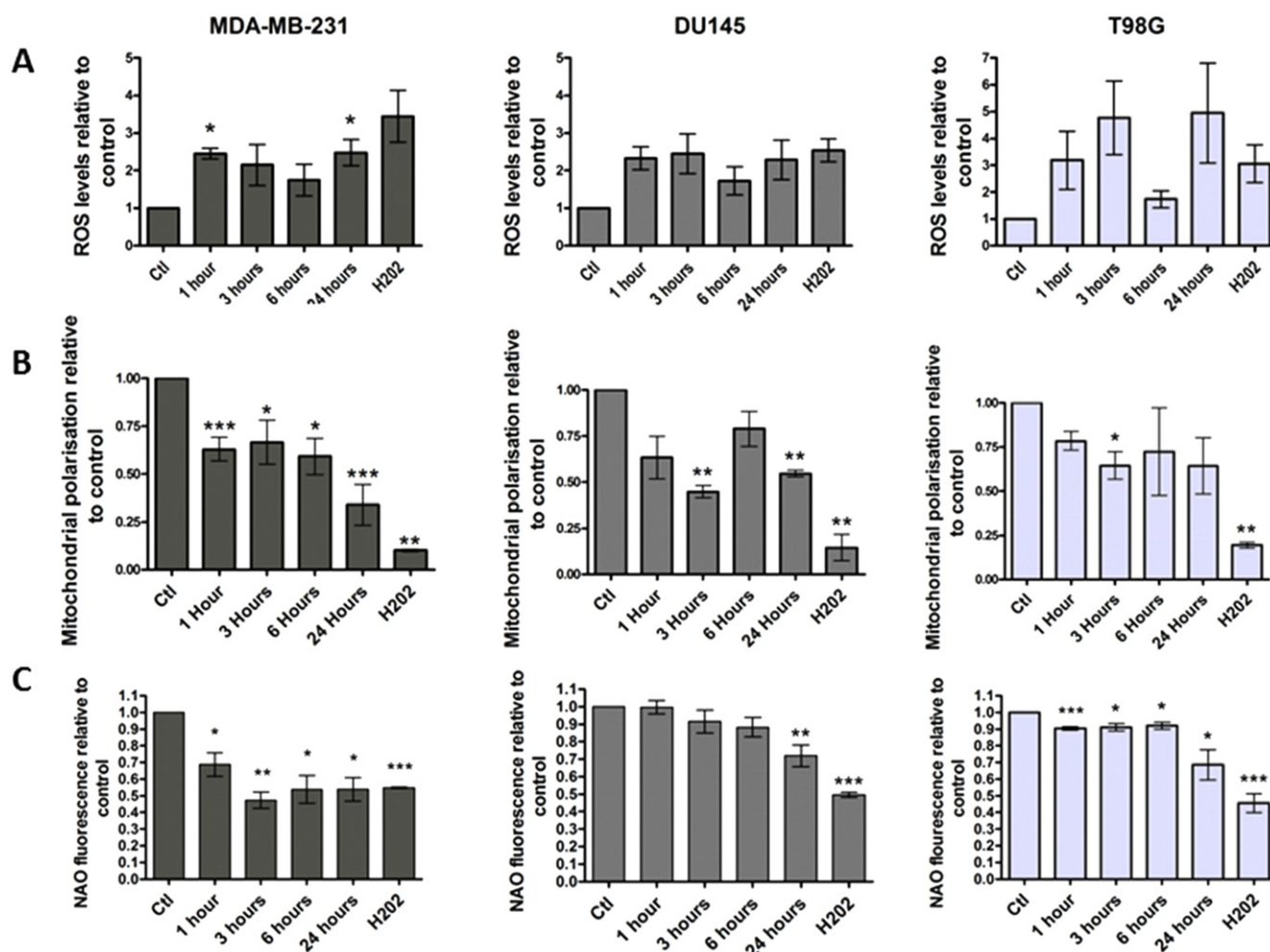


Figure 1. (A) Reactive oxygen species with GNP treatment. Carboxy-H2DCFDA was used to measure reactive oxygen species levels relative to untreated control using flow cytometry after cells were treated with 1.9 nm GNPs for indicated time points. Cells were treated with 2.5 mM H₂O₂ for 30 min as a positive control. $n = 4$. (B) Mitochondrial polarisation after GNP treatment. Mitochondrial polarisation was measured in MDA-MB-231, DU145 and T98G cells by TMRE fluorescent flow cytometry relative to untreated controls after cells were treated with 1.9 nm GNPs for indicated time points. Cells were treated with 2.5 mM H₂O₂ for 30 min as a negative control. $n = 4$. (C) Mitochondrial oxidation after GNP treatment. Mitochondrial oxidation as measured by Nonyl Acridine Orange flow cytometry relative to untreated control after cells were treated with 1.9 nm GNPs for indicated timepoints. Cells were treated with 2.5 mM H₂O₂ for 30 min as a positive control. $n = 4$. Means are presented \pm standard error of the mean. Significance was measured by paired t tests against controls. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

perchlorate (TMRE) dye using flow cytometry. TMRE is preferentially taken up by functional, polarised, negatively charged mitochondria causing cells to fluoresce red. In response to stress, mitochondria lose membrane potential and become fully permeable meaning the dye is no longer retained and fluorescence is lost. The effect of GNP exposure on mitochondrial membrane polarisation in MDA-MB-231, DU145 and T98G cells was assessed using TMRE and flow cytometry at 1, 3, 6 and 24 h time-points (figure 1(B)). The loss of TMRE fluorescence across all cell lines from as soon as 1 h exposure to 1.9 nm GNP indicates mitochondrial stress as a rapid response to GNP contact. Fluorescence is significantly reduced to 63% of control in MDA-MB-231 cells but non-significantly reduced to 63% and 78% in DU145 and T98G cells after 1 h exposure to 1.9 nm GNPs. These levels of fluorescence remain consistent in MDA-MB-231 and T98G cells throughout the time course with a further drop in

fluorescence to 34% at 24 h in MDA-MB-231 cells. Mitochondrial polarisation appears to fluctuate throughout the time course in DU145 cells with some apparent recovery in polarity at 6 h when TMRE fluorescence increases to 79%, however, at 24 h it has significantly reduced again to 54% of the control.

Levels of mitochondrial oxidation were evaluated using nonyl-acridine orange (NAO), a fluorescent dye with an affinity for cardiolipin; a lipid found in the inner mitochondrial membrane. Upon mitochondrial oxidation, this lipid is oxidised and NAO can no longer bind, thereby fluorescence is lost. Mitochondrial oxidation was measured after 1, 3, 6 and 24 h incubations with GNPs in MDA-MB-231, DU145 and T98G cells (figure 1(C)). After 1 h treatment with 1.9 nm GNPs, MDA-MB-231 and T98G cells undergo significant mitochondrial oxidation with a reduction in fluorescence of 32% and 10% from controls respectively. Both cell lines

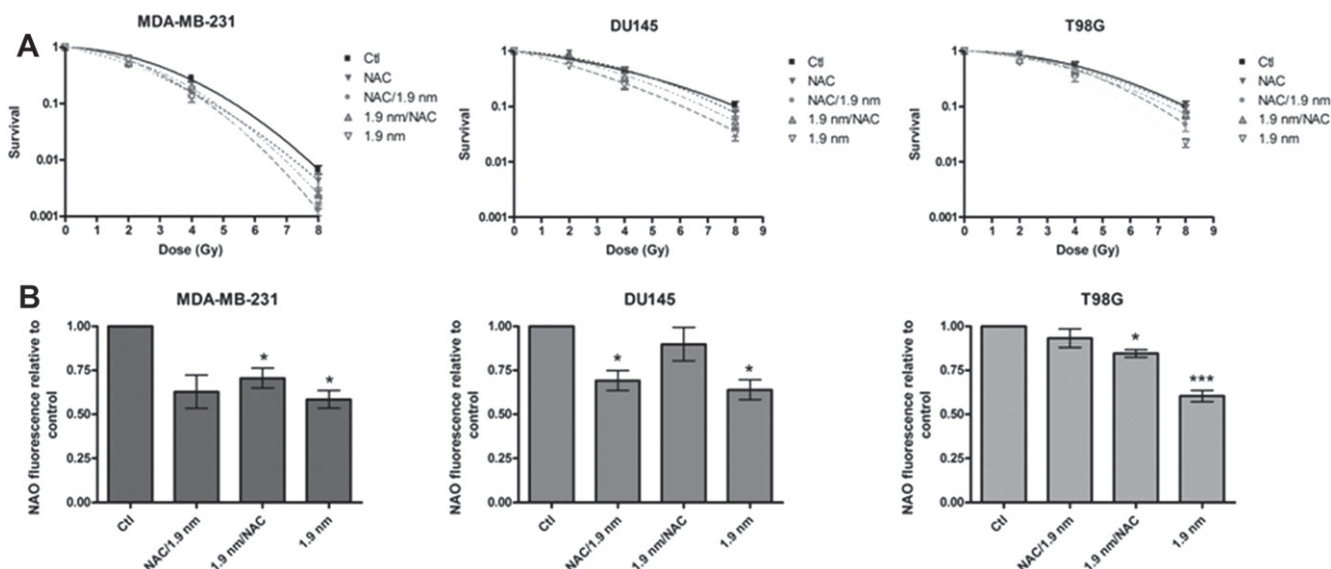


Figure 2. (A) Clonogenic cell survival curve with NAC and 1.9 nm GNPs. Cell survival following treatment with different combinations of anti-oxidant NAC and 1.9 nm gold nanoparticles along with irradiation. NAC/1.9 nm indicates cells were treated with 5 mM NAC for 2 h prior to $500 \mu\text{g ml}^{-1}$ 1.9 nm GNPs for 24 h and then irradiated. 1.9 nm/NAC indicates cells were treated for 24 h $500 \mu\text{g ml}^{-1}$ 1.9 nm GNPs prior to 2 h 5 mM NAC treatment and then irradiated. Means are presented \pm standard error of the mean. $n = 5$. (B) Mitochondrial oxidation after treatment with ROS scavenger. Mitochondrial oxidation as measured by Nonyl Acridine Orange flow cytometry relative to untreated control after cells were treated with an ROS scavenger (NAC) prior to or after 1.9 nm GNP exposure for 24 h. Means are presented \pm standard error of the mean. $n = 4$. Significance was measured by paired t tests. $* = p \leq 0.05$.

continue to undergo mitochondrial oxidation for the duration of the time course with a reduction in fluorescence of 47% in MDA-MB-231 cells and 40% in T98G cells at 24 h. DU145 cells however, do not undergo significant mitochondrial oxidation until 24 h GNP exposure with a significant reduction in fluorescence of 28%.

Cellular GNP uptake was also measured by inductively coupled plasmon mass spectrometry for this treatment time course (supplementary figure 3(A)). MDA-MB-231 and T98G cells showed similar GNP uptake with an increased in GNPs per cell as the time course elapsed. DU145 cells showed fewer GNPs per cell than MDA-MB-231 and T98G cells, but a similar increase in uptake as the time course progressed.

The impact of oxidative stress on cell survival post irradiation

In order to determine what impact, if any, this oxidative stress has on overall cell survival post irradiation with GNPs we used the antioxidant NAC to abrogate the oxidative stress. However, we found that the NAC was ineffective at preventing GNP-induced ROS levels at the point of radiation (24 h after NAC treatment)(supplementary figure 4(A)). Despite the presence of ROS with the NAC/1.9 nm combination treatment, mitochondrial polarisation was restored to near control levels across all cell lines (supplementary figure 4(B)) and NAC also prevented GNP-induced mitochondrial oxidation in certain treatment combinations in DU145 and T98G cells (figure 2(B)).

Clonogenic cell survival assays were then performed to determine the effect of these events on overall cell survival post irradiation (figure 2(A)). The DEFs for these treatments

are presented in table 1 alongside values corrected for the effects of NAC and irradiation alone. In MDA-MB-231 cells, the radiosensitising effect of 1.9 nm GNPs can be completely abrogated by treatment with NAC either prior to GNP exposure (NAC/1.9 nm) or post GNP exposure (1.9 nm/NAC) with DEFs at 2 Gy of 1.52 ± 0.03 with 1.9 nm GNPs alone, 1.01 ± 0.01 with NAC treatment prior to GNP exposure and 0.97 ± 0.02 with GNP exposure then NAC treatment. NAC treatment prior to GNP exposure also abrogated the radiosensitising effects of GNPs in T98G cells with a reduction in DEF at 2 Gy from 1.92 ± 0.05 with GNPs to 0.82 ± 0.05 with NAC followed by GNP treatment. In DU145 cells the radiosensitising effect is unaltered by NAC treatment prior to GNP exposure as significant radiosensitization still occurs with a DEF at 2 Gy of 2.02 ± 0.01 . Alternatively, treating DU145 cells with NAC post GNP exposure and prior to irradiation significantly protects against the radiosensitising effects of 1.9 nm GNPs reducing the DEF at 2 Gy from 1.82 ± 0.01 with GNPs alone to 1.33 ± 0.04 with GNPs then NAC. This protective effect becomes more prominent in DU145 cells with increasing dose with a DEF of 1.05 ± 0.01 at 8 Gy. Radiosensitization in T98G cells is also greatly reduced but not removed with DEF at 2 Gy of 1.15 ± 0.04 .

In order to ensure the observed effects were a result of the impact of NAC on biological mechanisms and not on cell uptake of GNPs, cellular GNP content after NAC and/or GNP treatment was measured by inductively coupled plasmon mass spectrometry (supplementary figure 3(B)). It was found that treating with NAC before or after 1.9 nm exposure did not significantly decrease GNP cellular content and in fact

Table 1. Dose enhancement factors. Table of DEFs with errors for cells treated with NAC and/or 1.9 nm GNPs at 2, 4 and 8 Gy. Numbers in *bold italics* are corrected for effect of NAC with radiation.

	MDA-MB-231			DU145			T98G		
	2 Gy	4 Gy	8 Gy	2 Gy	4 Gy	8 Gy	2 Gy	4 Gy	8 Gy
NAC	1.23 ± 0.01	1.24 ± 0.01	1.05 ± 0.01	0.81 ± 0.03	0.90 ± 0.01	1.02 ± 0.01	1.31 ± 0.05	1.12 ± 0.02	0.99 ± 0.01
NAC—1.9 nm	1.25 ± 0.01	1.19 ± 0.01	1.17 ± 0.01	1.64 ± 0.01	1.44 ± 0.01	1.28 ± 0.1	1.08 ± 0.05	1.12 ± 0.02	1.15 ± 0.01
	1.01 ± 0.01	0.96 ± 0.01	1.11 ± 0.01	2.02 ± 0.03	1.60 ± 0.01	1.25 ± 0.01	0.82 ± 0.07	1.00 ± 0.03	1.16 ± 0.01
1.9 nm—NAC	1.19 ± 0.02	1.14 ± 0.01	1.10 ± 0.01	1.08 ± 0.03	1.08 ± 0.01	1.07 ± 0.01	1.50 ± 0.04	1.19 ± 0.01	1.07 ± 0.01
	0.97 ± 0.02	0.92 ± 0.01	1.05 ± 0.01	1.33 ± 0.04	1.20 ± 0.03	1.05 ± 0.03	1.15 ± 0.06	1.06 ± 0.03	1.08 ± 0.04
1.9 nm	1.52 ± 0.03	1.27 ± 0.01	1.14 ± 0.01	1.82 ± 0.01	1.59 ± 0.01	1.36 ± 0.01	1.92 ± 0.05	1.53 ± 0.01	1.33 ± 0.01

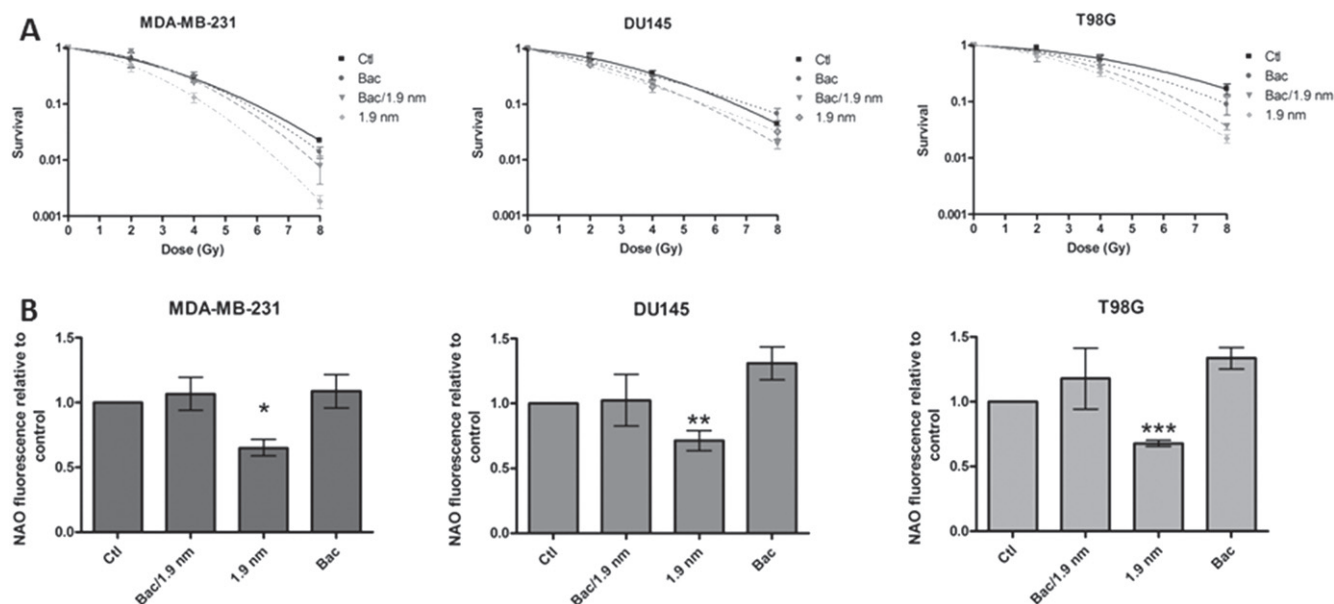


Figure 3. (A) Cell survival following bacitracin and GNP treatment combined with irradiation. Cells were treated with 3 mM bacitracin for 2 h and then $500 \mu\text{g ml}^{-1}$ 1.9 nm GNPs for 24 h, irradiated, re-seeded and left to form colonies for 7–9 d. Means are presented \pm standard error of the mean. $n = 5$. (B) Mitochondrial oxidation after combined bacitracin and GNP treatment. Mitochondrial oxidation was assessed by Nonyl Acridine Orange fluorescent flow cytometry after cells were treated with indicated concentration/incubation combinations of bacitracin followed by 1.9 nm GNP exposure for 24 h. Means are presented \pm standard error of the mean. $n = 4$. Significance was measured by paired t tests. * = $p \leq 0.05$, *** = $p \leq 0.001$.

resulted in significantly greater GNPs per cell in DU145 cells especially in the NAC pre-treated cells.

The role of protein disulphide isomerase (PDI)

Given the oxidative stress cell response upon treatment with GNPs and the knowledge that the GNPs are thiol-coated, (PDI) was identified as a potential mediator of the overall cellular response to GNPs. The enzymatic action of PDI is blocked by the antibiotic, bacitracin which has been shown to be a specific inhibitor of the reductive activity of PDI [21]. Having established that bacitracin had no effect on GNP-induced mitochondrial depolarisation (supplementary figure 5), its effect on mitochondrial oxidation and overall cell survival post GNP irradiation was measured (figure 3). Bacitracin effectively inhibited GNP-induced mitochondrial oxidation across all cell lines. DEFs for the clonogenic cell survival curves in figure 3 are presented in table 2 along with values corrected for the effects of bacitracin and radiation alone. The radiosensitising effect of GNPs is completely abrogated in MDA-MB-231 cells across all doses and significantly reduced in DU145 and T98G cells to 1.08 ± 0.03 and 1.17 ± 0.07 at 2 Gy respectively with bacitracin treatment.

Discussion

Oxidative stress manifested as elevated levels of ROS following exposure to GNPs has been described by numerous studies [18, 19, 22–25]. One of our previous studies showed an effect on mitochondrial function in response to GNPs but

this was not linked to radiation response [20]. Upon observation of an oxidative cellular response involving ROS and mitochondrial depolarisation and oxidation, it was important to understand the impact of each event on GNP radiosensitisation in order to elucidate the mechanism. Using NAC, we were able to establish that the presence of ROS had no effect on post irradiation cell survival, with NAC treatment ROS were still present in cells at the point of radiation, however, GNP radiosensitisation was reduced in MDA-MB-231 cells and entirely absent in T98G cells (supplementary figures 4(A) and 2).

After treatment with NAC prior to GNP exposure, mitochondrial membrane depolarisation was prevented across all cell lines; however radiosensitisation in DU145 cells was still observed. This suggests that mitochondrial depolarisation is not a driver in GNP radiosensitisation. Furthermore, the significant increase in GNP uptake seen in DU145 cells with NAC treatment did not result in an enhancement of radiosensitisation, supporting the hypothesis of a biological mechanism in addition to a physical mechanism in radiosensitisation. Using different combinations of NAC and GNP treatment, mitochondrial oxidation was prevented in both the DU145 and T98G cell lines prior to irradiation. When mitochondrial oxidation was prevented, radiosensitisation was significantly reduced suggesting mitochondrial oxidation is a key event in GNP radiosensitisation.

Given the overall oxidative stress response observed in cells exposed to GNPs and the fact that these GNPs are thiol-coated, PDI was identified as a potential driver in the overall cellular response. PDI is an enzyme that catalyses the creation and cleavage of disulphide bonds in folding proteins in the

Table 2. Dose enhancement factors. Table of DEFs with errors for cells treated with bacitracin and/or 1.9 nm GNPs at 2, 4 and 8 Gy. Numbers in ***bold italics*** are corrected for the effect of bacitracin with radiation.

	MDA-MB-231			DU145			T98G		
	2 Gy	4 Gy	8 Gy	2 Gy	4 Gy	8 Gy	2 Gy	4 Gy	8 Gy
Bac	0.92 ± 0.02	1.00 ± 0.02	1.06 ± 0.01	1.32 ± 0.02	1.09 ± 0.01	0.90 ± 0.01	1.18 ± 0.07	1.20 ± 0.04	1.18 ± 0.02
Bac /1.9 nm	0.91 ± 0.03	1.01 ± 0.03	1.14 ± 0.01	1.43 ± 0.02	1.28 ± 0.01	1.17 ± 0.01	1.39 ± 0.03	1.40 ± 0.02	1.40 ± 0.01
	<i>0.98 ± 0.04</i>	<i>1.04 ± 0.04</i>	<i>1.03 ± 0.01</i>	<i>1.08 ± 0.03</i>	<i>1.17 ± 0.01</i>	<i>1.30 ± 0.01</i>	<i>1.17 ± 0.07</i>	<i>1.16 ± 0.04</i>	<i>1.19 ± 0.01</i>
1.9 nm	1.52 ± 0.03	1.27 ± 0.01	1.14 ± 0.01	1.82 ± 0.01	1.59 ± 0.01	1.36 ± 0.01	1.92 ± 0.05	1.53 ± 0.01	1.33 ± 0.01

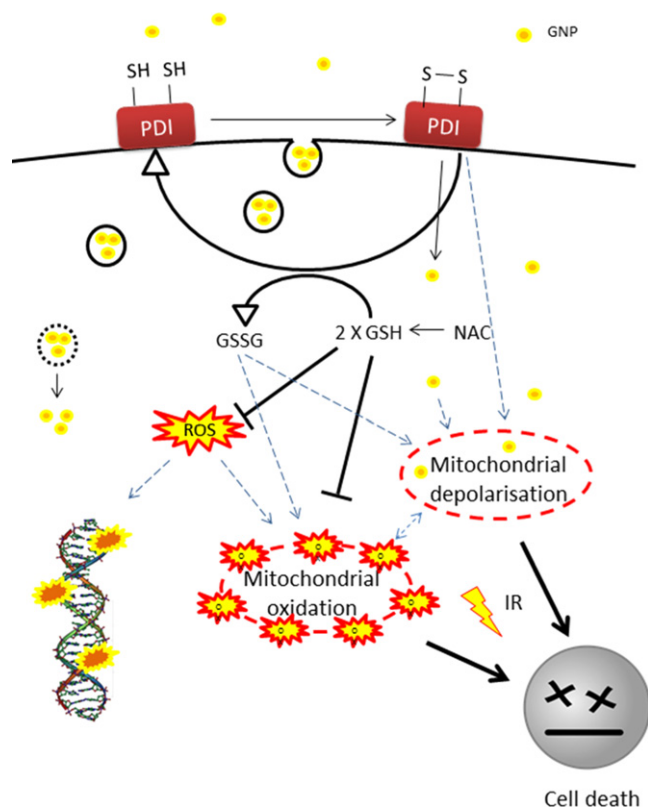


Figure 4. Mechanism of action. Proposed mechanism of action for the radiosensitising effect of 1.9 nm gold nanoparticles.

endoplasmic reticulum (ER). It is primarily found in the ER, however, it has been shown to be present in the nucleus, mitochondria, cytosol and on the cell surface [26–28]. Its expression has been linked to oncogenesis and cancer survival as well as metastasis, identifying PDI as a potential therapeutic target [29]. Its role in reducing disulphide bonds on the cell surface and its link to mitochondrial oxidative stress suggests PDI as a potential mediator in thiol-coated GNP cell response. Bacitracin, an inhibitor of PDI, has been shown to inhibit only the reductase activity of PDI and not affect the other functions of PDI such as the oxidation and rearrangement of disulphide bonds [30].

Within the current study, bacitracin effectively prevented GNP-induced mitochondrial oxidation across all cell lines (figure 3(A)). Additionally, bacitracin did not prevent GNP-induced mitochondrial depolarisation (supplementary figure 5), supporting previous results suggesting mitochondrial depolarisation is not a key factor in GNP radiosensitisation. The mitochondrial depolarisation observed with bacitracin and GNP combination treatment could be related to the role of PDI in preventing misfolded proteins. The formation of pores in the mitochondria membrane which cause a loss of polarisation have been linked to an accumulation of misfolded proteins within the mitochondrial membrane [31]. Therefore blocking PDI could drive this misfolded protein pore formation regardless of the impact of blocking PDI on potential biological signalling pathways.

On the whole, the results of this study allow the identification of a biological mechanism in GNP radiosensitisation.

Given the role of PDI and the thiol-coating of the GNPs, the response is potentially a result of a thiol imbalance in the cellular environment. The proposed mechanism of action for the radiosensitising effect of 1.9 nm GNPs is illustrated in figure 4. PDI–GNP interaction at the cell surface appears to be a key driver in the sensitisation of cells to radiation. It is possible that cell-surface PDI reduces the thiolated surface of the GNPs, resulting in PDI oxidation which would require reduction by glutathione. This reduction reaction would lead to the oxidation of glutathione to glutathione disulphide. Prolonged repetition of this process can result in depletion of the cellular antioxidant glutathione, causing a cellular redox imbalance and ultimately oxidative stress. The excess glutathione disulphide, cellular ROS and thiolated GNPs could react with the outer mitochondrial membrane resulting in the formation of pores in the mitochondrial membrane and therefore mitochondrial depolarisation. These agents can also oxidise mitochondria however, this appears to be dependent on cellular levels of glutathione which are enhanced by NAC treatment. This study has shown mitochondrial oxidation to be the most potent event in the radiosensitization of cells; however, particularly in MDA-MB-231 cells; mitochondrial depolarisation can also cause radiosensitization.

Disruptions in thiol metabolism have been attributed to the radiosensitising effects of inhibiting glucose metabolism by 2-Deoxy-D-glucose [32]. Moreover, downregulation of glutathione has been shown to radiosensitise cells in comparison to stimulation of its synthesis which has been shown to be radioprotective [33]. However, radiosensitization by the disruption of glutathione levels and overall cellular thiol metabolism through the activation of PDI has previously been described. Given the potential implications of PDI in cancer, it is possible that differential expression levels of PDI in cancer cells could explain the ranges of radiosensitisation observed with these GNPs.

It should also be noted that it is likely that GNP exposure may induce other cellular stresses which can also lead to sensitisation—as suggested by the residual sensitisation effects seen in some cell lines when the pathway described in this work is inhibited. This is an ongoing open research question in the field of nanoparticle radiosensitisation, and this work and others highlights the need to consider all aspects of a nanoparticle's characteristics to understand their sensitising properties.

The question of GNPs being radioenhancers or radiosensitisers is an important issue to address for their clinical use. Despite the attractiveness of a compound which can physically enhance radiation dose, this would be of restricted practicality in patient treatment due to clinical radiotherapy machines emitting in the megavoltage range and gold absorption being most efficient at dose enhancement in the kilovoltage range. As a result, GNPs which act as biologically driven radiosensitisers would be more applicable to clinical scenarios, as they would be expected to maintain their sensitising effects in megavoltage exposures. Additionally the concentrations of GNPs necessary for significant physical dose enhancement are potentially unachievable in patients; therefore a mechanism by which GNPs are effective in

combination with radiation at considerably lower concentrations is more clinically relevant. A final potential benefit of biological mechanisms of sensitisation may be improved specificity—if GNPs can be produced which specifically impact on pathways which are deregulated in cancer, sensitisation can be obtained in tumour cells while sparing normal tissue. Such an effect is seen in the 1.9 nm particles considered in this work, which have negligible sensitising effects in normal fibroblasts (supplementary information).

The GNPs in this study have been shown to act predominantly as radiosensitisers rather than radioenhancers. The calculations for physical dose enhancement presume an even distribution of the dose and GNPs throughout the cell. Although we know the dose will not be evenly distributed, this should only account for a minor overestimation of the dose enhancement and cannot justify the complete lack of radioenhancement when cells are treated with NAC or bacitracin. However, little is known about the effect of GNP distribution on these calculations. Many studies including those from our own group have found that GNPs aggregate within the cell, primarily in endosomes. The effect of these aggregates in terms of dose enhancement has not been investigated but has the potential to interfere with the overall physical effects. The discovery of this novel mechanism of radiosensitization through the use of GNPs highlights the role of nanotechnology in innovating research and the development of new therapeutics. The vast catalogue of nanoparticle combinations has the potential to significantly add to the knowledge of cellular mechanisms and identify novel targets for the therapy of various diseases not only through the use of nanoparticles as therapeutic agents themselves but also by studying the interactions of cells with different nanoparticles.

Conclusions

The data presented in this paper clearly demonstrate a biological mechanism which contributes significantly to the radiosensitisation observed with 1.9 nm GNPs. Irradiation with GNPs was shown to drive increased oxidative stress caused by the interaction of the GNPs with the cell membrane protein PDI, which in turn led to an overall disruption in the thiol balance of the cell, which in turn led to significant increases in cell killing. The action of GNP radiosensitization described in this paper identifies a novel mechanism for the radiosensitization of cancer cells as well as identifying new targets for sensitising cells to radiation in the form of PDI and the oxidation of mitochondria which could aid in the development of new radiosensitising agents.

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Authors' contributions

LET designed, performed and analysed laboratory experiments, and drafted the manuscript. SJM provided statistical support and intellectual contribution. KB helped draft the manuscript and supervised experiments. FJC, KMP and GS proposed experimental objectives and supervision. All authors made extensive intellectual contributions to the work and in reviewing of the manuscript.

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